Low-dose endobronchial gene transfer to ameliorate lung graft ischemia-reperfusion injury

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Objective: This study was undertaken to determine whether low-dose endobronchial transfer to the donor of the gene for human interleukin 10 would decrease ischemia-reperfusion injury in lung transplantation.

Methods: Experiments used male Fischer rats. Donor animals underwent right thoracotomy. A catheter was introduced into the left main bronchus, and vector was instilled. Group I (n = 6) received 2×10^7 plaque-forming units of adenovirus encoding human interleukin 10, group II (n = 6) received an adenovirus control encoding β-galactosidase, and group III (n = 6) received saline solution. After instillation the left main bronchus was clamped for 60 minutes. Lungs were removed 24 hours later and stored in low-potassium dextran glucose solution for 18 hours before left lung transplantation. Graft function was assessed at 24 hours immediately before the animals were killed. Ratio of wet to dry weight and tissue myeloperoxidase activity were measured. Transgenic expression of human interleukin 10 was evaluated by means of enzyme-linked immunosorbent assay and immunohistochemical assay.

Results: Arterial oxygenation was significantly improved in group I relative to groups II and III (257.6 ± 59.7 mm Hg vs 114.6 ± 66.9 mm Hg and 118.6 ± 91.1 mm Hg, $P = .008$ and $P = .007$, respectively). Neutrophil sequestration, as measured by myeloperoxidase activity, was also significantly reduced in group I relative to groups II and III (0.141 ± 0.025 vs 0.304 ± 0.130 and 0.367 ± 0.153 Δ optical density units/[min · mg protein], $P = .029$ and $P = .004$, respectively). Enzyme-linked immunosorbent assay and immunohistochemical assay demonstrated the expression of human interleukin 10 in transfected lungs only.

Conclusions: Low-dose endobronchial transfer to the donor of the gene for human interleukin 10 ameliorated ischemia-reperfusion injury in rodent lung transplantation by improving graft oxygenation and reducing neutrophil sequestration. Only 2×10^7 plaque-forming units of adenoviral vector were required for functional transgenic expression. Endobronchial gene transfer to lung grafts may be a useful delivery route even at low doses.

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Lung transplantation is a well-established treatment for end-stage chronic lung disease.[1] Two major complications of lung transplantation that may result in graft dysfunction are ischemia-reperfusion injury and graft rejection. Although these complications have been studied extensively, they continue to adversely affect outcome, with no definitive therapy established to date.[2]

Gene therapy has emerged as a new strategy for investigation in the field of thoracic surgery. It has been applied to the areas of lung transplantation,[3] malignant disease,[4] and cystic fibrosis.[5] Our laboratory has demonstrated the feasibility of gene therapy in lung transplantation through the use of a rodent lung transplantation model.

In general there are several strategies for using gene therapy in lung transplantation. Variations include the type of vector (naked plasmid, liposome, adenovirus, etc), ex vivo or in vivo gene transfer, transfer to the donor or recipient, and systemic or localized delivery routes. Endobronchial gene therapy for the lung graft is an excellent localized strategy for the treatment of such transplant-related insults as ischemia-reperfusion injury and graft rejection. Endobronchial transfection has been performed for acute rejection,[6] but no studies to date have shown a functional improvement in ischemia-reperfusion injury from the use of endobronchial transfection.

Interleukin 10 (IL-10) is a potent anti-inflammatory cytokine that is produced mainly by macrophages and T_H2 cells.[7] It has been studied for a variety of inflammatory injuries and may reduce the ischemia-reperfusion injury after lung transplantation.[7] The goal of this study was to determine whether low-dose endobronchial transfection of the gene for IL-10 to the donor lung could decrease lung graft ischemia-reperfusion injury after prolonged cold ischemia.

Materials and Methods

Animals

Fischer 344 rats (Harlan Sprague Dawley, Inc, Indianapolis, Ind), weighing 250 to 280 g were used in all experiments. All animal procedures were approved by the Animal Studies Committee at Washington University in St Louis. Animals received humane care in compliance with "Principles of Laboratory Animal Care" formulated by the National Society for Medical Research and the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences and published by the National Institutes of Health (NIH publication 85-23, revised 1985).

Adenoviral Vectors

AdRSVhIL-10 is a replication-deficient recombinant adenoviral vector encoding the gene for human IL-10 (hIL-10). It was generated as a homologous recombinant between pAdRSVhIL-10 and human adenovirus serotype 5 derivative d1309 by standard methods.[8] The bioactivity of this recombinant adenovirus has been confirmed by in vitro bioassay.[9] The adenovirus used in this study was purchased from the Gene Therapy Vector Core, College of Medicine, University of Iowa, Iowa City, Iowa.

First-generation replication-deficient adenovirus serotype 5 carrying the *Escherichia coli lacZ* gene, which encodes for β-galactosidase, driven by the constitutive cytomegalovirus promoter (Ad5CMVLacZ). The gene for β-galactosidase is a nonfunctional reporter gene used as an adenovirus control. It was purchased from the Gene Therapy Center at the University of North Carolina, Chapel Hill, NC.

Adenoviral amplification was performed by propagation in 293 cells to obtain high-titer stocks, as determined by the plaque assay (courtesy of Drs R. Jude Samulski and Douglas McCarty, Gene Therapy Center Vector Core Facility, University of North Carolina, Chapel Hill, NC). Purified viral aliquots were stored at –80°C in 10% glycerol buffered with 10-mmol/L tris(hydroxymethyl)aminomethane, 140-mmol/L sodium chloride, and 1-mmol/L magnesium chloride. These stocks were thawed and diluted in 0.1 mL sterile isotonic sodium chloride solution immediately before use.

Experimental Design

Fischer 344 rats were divided into six groups according to the transfecting vector. Group I (n = 6) and group IV (n = 6) donors received 2×10^7 plaque-forming units (pfu) of adenovirus encoding hIL-10, group II (n = 6) and group V (n = 3) donors received 2×10^7 pfu adenovirus control encoding β-galactosidase, and group III (n = 6) and group VI (n = 3) donors received isotonic sodium chloride solution. The dose of 2×10^7 pfu was chosen on the basis of preliminary experiments (data not shown). Transfection with 2×10^6 pfu did not improve lung graft oxygenation, whereas transfection with more than 1×10^8 produced macroscopic lung edema and atelectasis. Endobronchial gene transfection was performed as follows. Animals were anesthetized with a subcutaneous injection of ketamine hydrochloride (25 mg/kg) and atropine sulfate (0.25 mg/kg). After endotracheal intubation with a 14-gauge catheter, animals received mechanical ventilation with a small-animal Harvard ventilator (tidal volume 2.5 mL, respiratory rate 60 breaths/min) with 0.75% halothane and 99.25% oxygen. Donors underwent right thoracotomy. A catheter was introduced through the endotracheal tube into the left main bronchus, and vector diluted in 0.1 mL sterile isotonic sodium chloride solution was instilled. After 10 minutes of bilateral ventilation following instillation, the left main bronchus was clamped for 60 minutes at the end-inspiratory phase of mechanical ventilation. After the left main bronchus was unclamped, the right thoracotomy was closed and a temporary chest tube was placed. The chest tube was removed after recovery from anesthesia.

Lungs were harvested 24 hours after gene transfection. Briefly, after general anesthesia with intraperitoneally administered pentobarbital (65 mg/kg), mechanical ventilation, systemic heparinization (300 units), and median laparosternotomy, donor rat lungs were flushed through the main pulmonary artery with 20 mL cold (4°C) low-potassium dextran glucose solution at 20 cm H_2O pressure. The heart-lung block was then removed with the lungs inflated at end-tidal volume.

In groups I, II, and III, the left lung was isolated and stored at 4°C in low-potassium dextran glucose until implantation. After 18 hours of preservation, recipient animals were anesthetized, intubated with a 14-gauge catheter, and had a left thoracotomy performed. The pulmonary vessels and bronchus were anastomosed with a modification of the previously described "cuff technique."[10] In groups IV, V, and VI, grafts were harvested from donors and evaluated for transgenic expression.

Graft Assessment

After 24 hours of reperfusion, recipient animals in groups I, II, and III were reanesthetized with pentobarbital as described previously. After tracheostomy, animals received mechanical ventilation with 100% oxygen. The right main bronchus and pulmonary artery were clamped to isolate the left lung graft. Animals were ventilated for 5 minutes at a tidal volume of 1.5 mL, respiratory rate of 100 breaths/min, and positive end-expiratory pressure of 1.0 cm H_2O, followed by arterial blood gas analysis with blood samples obtained from the ascending aorta. Recipients were then killed immediately, and the lungs were flushed with 20 mL cold (4°C) saline solution. Each left lung graft was isolated from the heart-lung block and divided into sections. The middle third of each lung graft was frozen in liquid nitrogen for myeloperoxidase activity measurement. The lower third of each graft was weighed, dried at 80°C for 48 hours, and then reweighed for calculation of the wet to dry weight ratio.

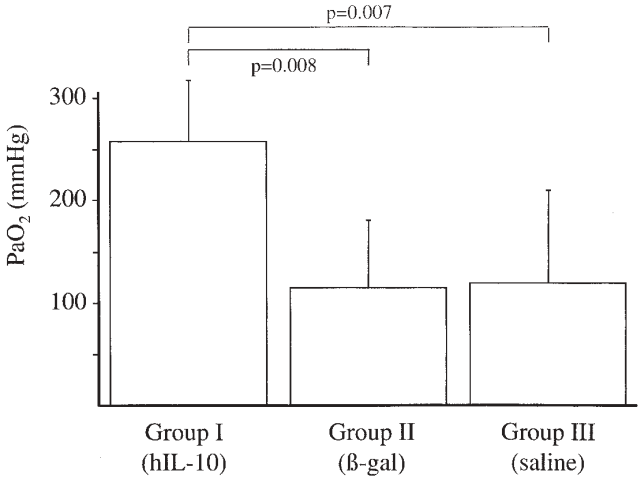

Figure 1. Mean arterial oxygen tension of isograft with inhaled 100% oxygen under right hilar crossclamping. Group I (transfected with gene for hIL-10) had Pao_2 superior to those of groups II (transfected with gene for β-galactosidase) and III (saline control). Differences between group I and groups II and III (257.6 ± 59.7 vs 114.6 ± 66.9 and 118.6 ± 91.1 mm Hg, respectively) were statistically significant. *Bar heights* represent mean; *error bars* represent SD.

Transgenic Expression of hIL-10

Transgenic expression of hIL-10 was evaluated in groups IV, V, and VI with enzyme-linked immunosorbent assay (ELISA) and immunohistochemical assay.

Myeloperoxidase Activity

Quantitative myeloperoxidase activity was determined as previously described.[11] Optical density was measured at 460 nm with a spectrophotometer (model PMQ II; Carl Zeiss, Oberkochen, Germany). Color development was linear from 5 minutes to 20 minutes. One unit of enzyme activity was defined as 1.0 optical density units per minute per milligram of tissue protein at room temperature.

ELISA

Lung specimen samples were homogenized with a lysis solution consisting of 100 mmol/L of potassium phosphate dibasic, pH 7.8, including 0.2% of Triton X-100 (Fisher Scientific Worldwide, Laboratory Projects Division, Springfield, NJ), and the protease inhibitor Complete Mini (Hoffmann-LaRoche Inc, Nutley, NJ). After 15 minutes of extraction at room temperature, the extraction was clarified by centrifugation at 11,000*g* for 10 minutes at 4°C and stored at –80°C until measurement. The hIL-10 ELISA kit was purchased from R&D Systems, (Minneapolis, Minn), and there is no cross-reactivity between hIL-10 and rat IL-10 proteins. Optical density was determined with an Ultra Microplate Reader (EL808; Bio-Tek Instruments, Inc, Winooski, Vt) set at 450 nm. The wavelength correction was performed under subtraction readings at 540 nm from the readings at 450 nm. The extract was subsequently assayed for total soluble protein (picograms per milligram total protein) by the method of Pierce Laboratories (Rockford, Ill).[12]

Immunohistochemical Assay

Tyramide Signal Amplification Biotin System kits (NEN Life Science Products, Inc, Boston, Mass) were used for immunohistochemical assay. Briefly, lungs were perfused with 20 mL isotonic sodium chloride solutions and 20 mL HistoChoice (Amresco Inc, Solon, Ohio). Specimens were fixed, cut, mounted, deparaffinized, and then treated with Dako Target Retrieval Solution (DAKO Corporation, Carpinteria, Calif) followed by 3% hydrogen peroxide in methanol. The sections were then incubated with an FcγII receptor blocker (purified anti–rat CD32; BD PharMingen, San Diego, Calif) and with Super Block Blocking Buffer (Pierce Chemical Company, Rockford, Ill) including 1% bovine serum albumin and 1% normal goat serum, followed by TNB Blocking Buffer in Tyramide Signal Amplification kits. After 60 minutes at room temperature the sections were incubated with a biotinylated rat anti–human IL-10 and viral IL-10 monoclonal antibody (BD PharMingen) at 1:25 dilution in TNB Blocking Buffer at 4°C overnight. They were then incubated with streptavidin–horseradish peroxidase followed by biotinyl tyramide solution. The streptavidin–horseradish peroxidase and biotinyl tyramide solution procedures were repeated. The slides were incubated with streptavidin–alkaline phosphatase (NEN Life Science Products). Specific binding was detected with the BCIP/NBT substrate working solution (Vector Laboratories, Inc, Burlingame, Calif) containing 5-mmol/L levamisole (Vector Laboratories) until detection of staining. The slides were then counterstained with nuclear fast red, dehydrated, mounted, and coverslipped.

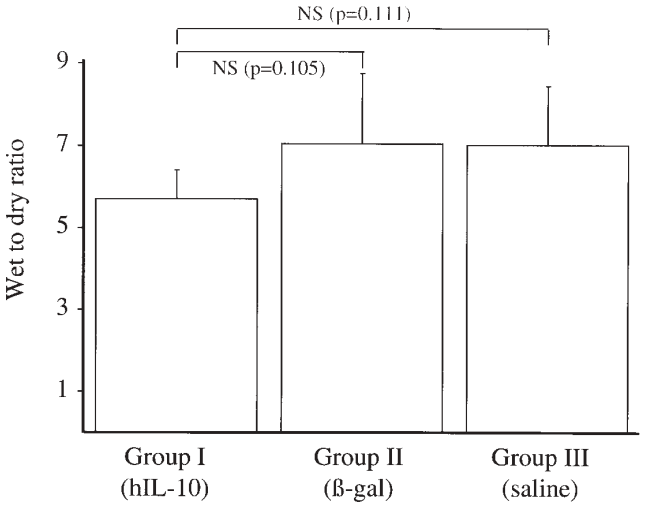

Figure 2. Wet to dry ratio of isograft. A trend toward significance existed in differences between mean wet to dry weight ratios in group I (transfected with gene for hIL-10) and in groups II (transfected with gene for β-galactosidase) and III (saline control). Ratios of groups I, II, and III were 5.69 ± 0.71, 7.04 ± 1.73, and 7.01 ± 1.43, respectively. *Bar heights* represent mean; *error bars* represent SD.

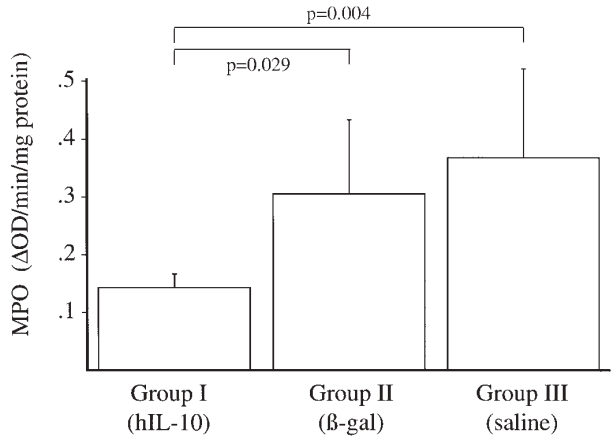

Figure 3. Myeloperoxidase of reperfused lung isograft. Myeloperoxidase activity, reflected by tissue neutrophil sequestration and immigration, was significantly reduced in group I (transfected with gene for hIL-10) relative to groups II (transfected with gene for β-galactosidase) and III (saline control). Activities were 0.141 ± 0.025, 0.304 ± 0.130, and 0.367 ± 0.153 Δ optical density units (OD)/min · mg protein. *Bar heights* represent mean; *error bars* represent SD.

Statistical Analysis

All values are described as mean ± SD. Data not normally distributed were analyzed after logarithmic correction. One-way analysis of variance with pairwise comparison by the Fisher positive least significant difference method was used to compare overall differences among groups.

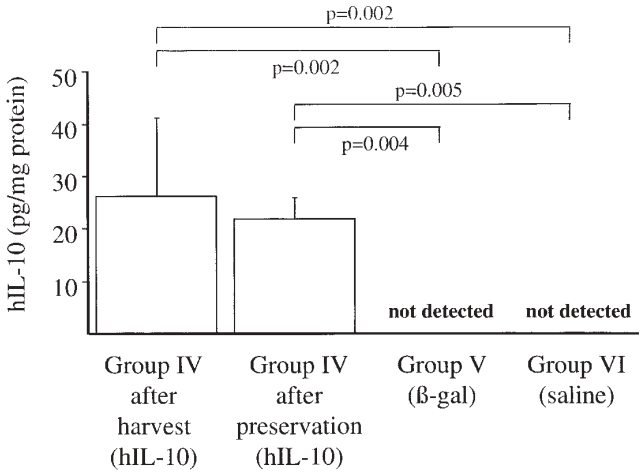

Figure 4. ELISA for hIL-10 in lung isograft without reperfusion. Superior expression of hIL-10 was observed in 3 hIL-10–transfected lung specimens obtained just after explantation (26.2 ± 15.2 pg/mg total protein for group IV, transfected with gene for hIL-10) but was not detected in groups V (transfected with gene for β-galactosidase) and VI (saline control). Statistically significant differences existed between group IV and groups V and VI. Three specimens from group IV lungs transfected with hIL-10 were preserved 18 hours after explantation and were also used for measurement; their value was 21.7 ± 4.1 pg/mg total protein. *Bar heights* represent mean; *error bars* represent SD.

Results

Isolated Lung Isograft Gas Exchange

Pao_2 levels for group I transfected with hIL-10 were significantly higher than in groups II and III (Figure 1). Mean $Paco_2$ levels in group I were significantly lower than in group II but not in group III (28.2 ± 6.7 vs 41.9 ± 8.6 and 35.9 ± 11.0 mm Hg, P = .018 and .158, respectively, by analysis of variance).

Wet to Dry Weight Ratio

The wet to dry ratio is an indicator of tissue edema. Group I lungs had lower wet to dry ratios, reflecting less tissue edema, than either group II or III lungs (Figure 2).

Myeloperoxidase Activity

Myeloperoxidase activity is a reflection of tissue neutrophil sequestration.[13] Myeloperoxidase levels were significantly reduced in group I lungs (Figure 3).

ELISA for hIL-10

The extracts derived from lung homogenate centrifugation were used for the measurement of hIL-10 in lung tissue.

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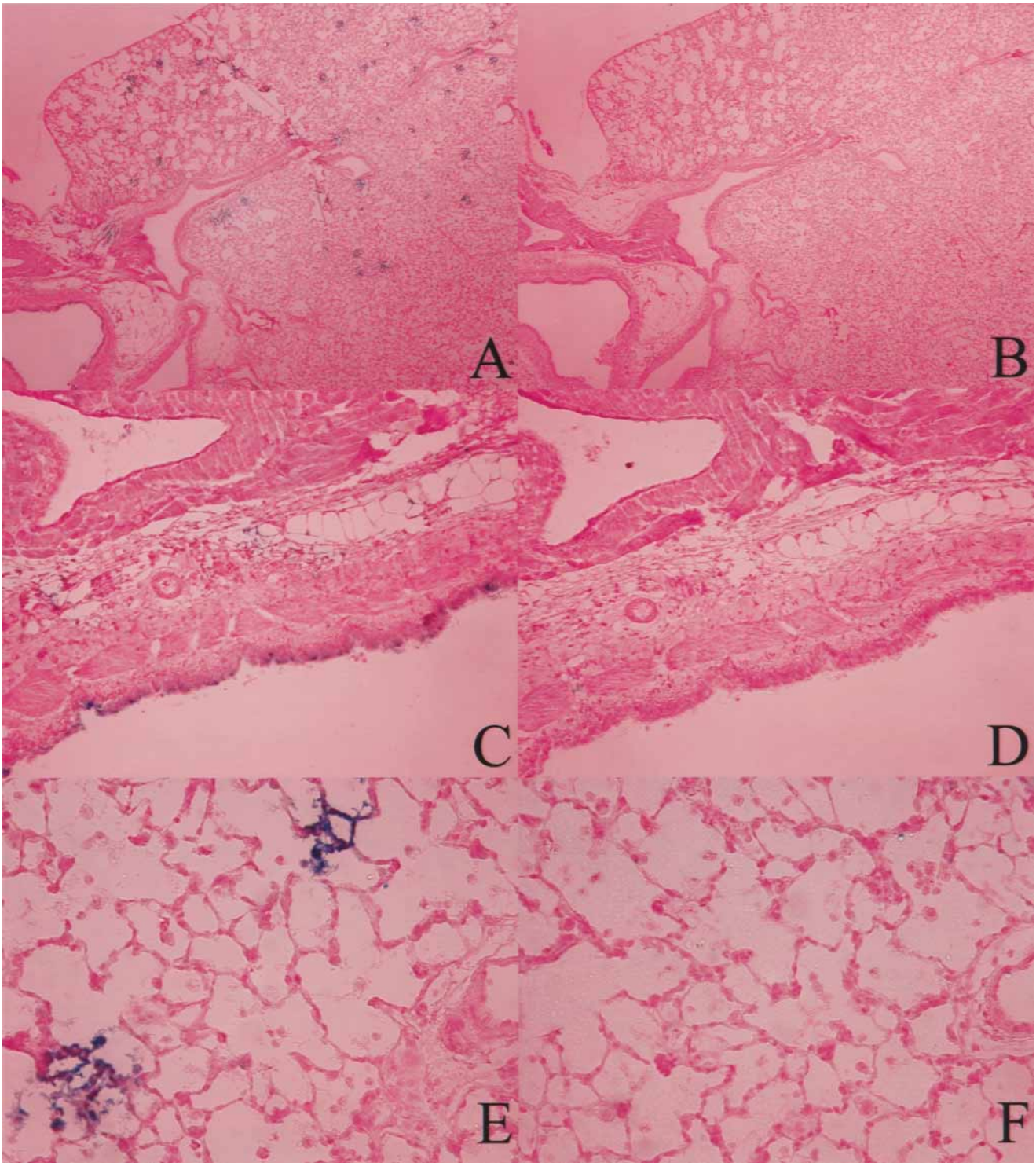

Figure 5. Immunohistochemical assay for hIL-10 in rat lungs without reperfusion. Characteristic patchy staining was observed in the alveolar areas of hIL-10–transfected lungs (A, magnification ×20; E, magnification ×200). Bronchial epithelial, bronchiolar epithelial, and alveolar cells were stained, but vessel endothelial, smooth muscle, and interstitial tissue cells were not stained (C, magnification ×100). B, D, and F are cells stained without cytokine antibodies that served as negative controls.

These lung specimens were not reperfused but stored at –80°C just after harvest. Transgenic expression of hIL-10 was observed in group IV lung specimens, which were obtained just after harvest and also after 18 hours of preservation (Figure 4).

Immunohistochemical Assay for hIL-10

Positive immunohistochemical staining for hIL-10 was observed in hIL-10–transfected lungs. Alveolar areas showed patchy staining (Figure 5, *A* and *E*). The distribution of expression was increased in the hilar area relative to the peripheral lung. Staining could be seen in bronchial epithelial, bronchiolar epithelial, and alveolar cells but was not seen in vessel endothelial, smooth muscle, or interstitial tissue cells (Figure 5, *C*). As expected, no hIL-10 staining was present in lungs transfected with either β-galactosidase or saline solution, because these vectors did not contain the gene for hIL-10.

Discussion

The best method of delivering gene therapy in lung transplantation remains to be determined. Both systemic and local strategies have been demonstrated to reduce acute lung injury.[6,13-17] Intravenous and intramuscular transfection are systemic delivery routes. Endobronchial, intra–pulmonary artery and intra–pulmonary vein transfection are localized delivery routes. Our laboratory has reduced acute rejection with both systemic[13] and localized[6,14] gene delivery. We have also demonstrated that systemic intravenous transfection of functional genes for heat shock protein 70,[15] endothelial nitric oxide synthase,[16] and hIL-10[17] to the donor attenuates ischemia-reperfusion injury. Our experience to date has shown that intravenous gene delivery requires at least 5×10^9 pfu of adenoviral vector. The efficacy of localized endobronchial gene transfer in reducing ischemia-reperfusion injury has not been previously demonstrated. In this study endobronchial hIL-10 delivery with a low dose of an adenoviral vector (2×10^7 pfu) significantly reduced ischemia-reperfusion injury. Endobronchial gene transfer thus reduced the quantity of vector needed 250-fold.

The pathophysiology of ischemia-reperfusion injury has been under intense investigation. The numerous pathologic factors involved include endothelial cell injury, neutrophil activation, platelet activation, oxygen-derived free radicals, and various cytokines.[18,19] IL-10 is a potent anti-inflammatory cytokine that is secreted primarily by macrophages and T_H2 cells.[7] Recent reports indicate that recombinant IL-10 reduces ischemia-reperfusion injury in the hind limb,[20] heart,[21] and lung.[22] This cytokine is a key factor in the ischemia-reperfusion injury cascade.

In this study overexpression of hIL-10 resulted in physiologic lung graft improvement and reduced ischemia-reperfusion injury. The mechanism for this improvement may be that gene transfer produced hIL-10 protein, which in turn led to a favorable anti-inflammatory environment. In assessment of the isolated lung graft, gas exchange is the most important and notable parameter. Lung graft oxygenation was almost doubled in hIL-10–transfected lungs relative to control lungs. In addition, hIL-10–transfected lungs demonstrated significantly lower neutrophil sequestration, as shown by myeloperoxidase activity. Other data, such as carbon dioxide levels and wet to dry ratio measurements, revealed trends toward physiologic improvement in hIL-10–transfected lungs.

Gene transfection was performed in vivo to the donor lung to enhance hIL-10 expression during cold ischemia and reperfusion. Ex vivo transfection is easier to perform than in vivo transfection, similar to performing a procedure on the side table in the clinical setting. However, we have previously demonstrated that ex vivo transfection followed by cold preservation does not produce gene expression at the time of reperfusion.[23] Because reperfusion injury begins immediately, ex vivo transfection is not beneficial in this setting. Ex vivo transfection should therefore continue to be considered as a potential strategy for the reduction of graft rejection[14] but not for amelioration of ischemia-reperfusion injury.

Cassivi and colleagues[24] revealed the potential for endobronchial gene therapy in lung transplantation. In their study in vivo endobronchial transfection of the donor produced β-galactosidase protein expression both at the time of reperfusion and 2 hours after transplantation. According to ELISA data in our study, hIL-10 protein was successfully expressed in lungs transfected with hIL-10 at the time of removal and 18 hours after cold preservation. Therefore the hIL-10 protein expressed in the donor before explantation would remain intact during cold storage and would be effective at the start of graft reperfusion, when reperfusion injury begins. The in vivo protocol used in this experiment transfected the donor 24 hours before lung removal. This is clinically feasible for living-related donor transplantation but is not ideal for most conventional lung transplants.

Systemic gene delivery requires large amounts of vector, and the systemic side effects are not clear. This study demonstrates that localized endobronchial gene delivery requires significantly less adenovirus vector. Endobronchial gene delivery is a uniquely graft-targeted transfection strategy for lung transplantation. The merits of endobronchial transfection are that it is easy to perform, may be minimally invasive, and only transfects the lung. Endobronchial gene transfer probably works in a paracrine manner. It transfects bronchial epithelium without significant systemic expression in heart, liver, native right lung, or plasma.[6,24,25] Endobronchial instillation to lung grafts thus may provide an effective, organ-selective transfection system for lung grafts.

Potential complications of endobronchial gene delivery include heterogeneous distribution of gene expression and local inflammation.[26] Immunohistochemical results in this study showed greater distribution of gene expression in the hilar area than in the peripheral area, with patchy alveolar staining. The heterogeneous distribution of gene expression probably reflects heterogeneous transfection. An aerosomal vector solution may provide an answer to the heterogeneous distribution problem, and studies are ongoing to develop this method. Local inflammation may be due to the heterogeneous volume of vector solution or direct toxicity of the adenovirus vector,[27] or gene transfer with adenoviral vectors may incite a host immune response, resulting in dose-dependent inflammation.[26] It is possible that the transfected lung would have an inflammatory response, such as pneumonia, atelectasis, or edema, after endobronchial transfection. In fact, some inflammatory cells in the alveoli were observed in our immunohistochemical slides.

The protein expression of the transfected gene is also dose dependent.[28] In this study we improved the expression of hIL-10 by clamping the bronchus during adenovirus instillation. Previous work in our laboratory indicates that clamping the bronchus during endobronchial transfection improves gene expression. The mechanism by which bronchus clamping can increase gene expression was found to be increased exposure time.[29] We speculate that clamping the bronchus reduces the amount of vector that would be exhaled, increases epithelial contact with the vector by decreasing respiratory movements, increases lung surface area by maintaining lung hyperinflation, and increases positive airway pressure at end-inspiration to promote adenoviral vector uptake by airway epithelial cells. In this study, graft function did not improve when 60 minutes of left main bronchus clamping was omitted (data not shown). We therefore believe that clamping the bronchus is an important part of this transfection strategy. Endobronchial transfection with this technique reduced the amount of adenovirus vector needed to a 250th of that needed with intravenous transfection.[15-17] Using less vector decreases costs, reduces local inflammation, and reduces viral antigenicity in cellular and humoral immune responses.[30] The balance between efficient gene transfection and airway inflammation warrants further investigation.

In the study of gene transfer for lung transplantation, the connection between gene expression and physiologic improvement is crucial. Transgenic expression without physiologic improvement probably occurs with low expression of transgenic proteins. However, the increased quantity of vector required for high protein expression increases vector-related side effects and thus may not lead to physiologic improvement. We therefore continue to search for the gene transfection strategy with the fewest and least severe side effects, the highest transfection efficiency, and the highest production of functional protein resulting in physiologic improvement so that we eventually may apply this strategy in the clinical setting.

In conclusion, in vivo endobronchial transfection of the gene for hIL-10 in the donor lung ameliorates ischemia-reperfusion injury in rodent lung transplantation. Lung grafts transfected with the gene for hIL-10 showed improved graft oxygenation and reduced neutrophil sequestration. In addition, the localized endobronchial transfection strategy used 250-fold less vector than other reported routes of adenovirus-mediated gene transfer. Endobronchial gene transfer to lung grafts is a potential strategy for clinical lung transplantation.

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